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Dynamic remodelling of human 7SK snRNP controls the nuclear level of active P-TEFb

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The 7SK small nuclear RNA (snRNA) regulates RNA polymerase II transcription elongation by controlling the protein kinase activity of the positive transcription elongation factor b (P-TEFb). In cooperation with HEXIM1, the 7SK snRNA sequesters P-TEFb into the kinase-inactive 7SK/HEXIM1/P-TEFb small nuclear ribonucleoprotein (snRNP), and thereby, controls the nuclear level of active P-TEFb. Here, we report that a fraction of HeLa 7SK snRNA that is not involved in 7SK/HEXIM1/P-TEFb formation, specifically interacts with RNA helicase A (RHA), heterogeneous nuclear ribonucleoprotein A1 (hnRNP), A2/B1, R and Q proteins. Inhibition of cellular transcription induces disassembly of 7SK/HEXIM1/P-TEFb and at the same time, increases the level of 7SK snRNPs containing RHA, hnRNP A1, A2/B1, R and Q. Removal of transcription inhibitors restores the original levels of the 7SK/HEXIM1/P-TEFb and '7SK/hnRNP' complexes. 7SK/HEXIM1/P-TEFb snRNPs containing mutant 7SK RNAs lacking the capacity for binding hnRNP A1, A2, R and Q are resistant to stress-induced disassembly, indicating that recruitment of the novel 7SK snRNP proteins is essential for disruption of 7SK/HEXIM1/P-TEFb. Thus, we propose that the nuclear level of active P-TEFb is controlled by dynamic and reversible remodelling of 7SK snRNP.

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Introduction

The human 7SK RNA is an abundant, RNA polymerase (RNAP) III-synthesized, small nuclear RNA (snRNA) (Wassarman and Steitz, 1991) (Figure 1A). The 7SK snRNA functions as a key regulator of cellular mRNA production by controlling the activity of the positive transcription elongation factor b (P-TEFb) (Garriga and Grana, 2004; Barboric and Peterlin, 2006; Peterlin and Price, 2006; Zhou and Yik, 2006). P-TEFb is a cyclin-dependent kinase that controls the elongation phase of transcription through phosphorylation of the C-terminal domain (CTD) of RNAPII at serine 2 (Price, 2000; Garriga and Grana, 2004). This phosphorylation event is essential for productive transcription elongation and also for coupling pre-mRNA synthesis with splicing and polyadenylation (Sims *et al*, 2004; Aguilera, 2005; Peterlin and Price, 2006; Zhou and Yik, 2006). The predominant form of human P-TEFb is composed of cyclin-dependent kinase 9 (Cdk9) and the regulatory subunit cyclin T1 (CycT1) (Price, 2000).

In exponentially growing HeLa cells, about 50% of P-TEFb is present in active form and it is likely associated with the positive regulator bromodomain protein 4 (Jang *et al*, 2005; Yang *et al*, 2005). The other half of P-TEFb exists in a catalytically inactive small nuclear ribonucleoprotein (snRNP) that, in addition to the hexamethylene bisacetamide-inducible proteins HEXIM1 and/or HEXIM2 (Michels *et al*, 2003; Yik *et al*, 2003, 2005; Byers *et al*, 2005), also contains the 7SK snRNA (Nguyen *et al*, 2001; Yang *et al*, 2001). Sequestering of P-TEFb into the kinase-inactive 7SK/HEXIM/P-TEFb complex is supported by multiple intermolecular interactions. First, the HEXIM1 and HEXIM2 proteins, in homo- or heterodimeric forms (Dulac *et al*, 2005; Li *et al*, 2005; Yik *et al*, 2005), bind to a distal region of the 5′-hairpin of 7SK snRNA (Egloff *et al*, 2006). Docking of 7SK induces a conformational change in HEXIM proteins that enables their acidic C-terminal region to interact with CycT1 (Michels *et al*, 2004; Yik *et al*, 2004; Barboric *et al*, 2005; Blazek *et al*, 2005; Schulte *et al*, 2005). Finally, recruitment and inactivation of P-TEFb *in vivo* necessitates another RNA–protein interaction formed between the 3′-hairpin of 7SK snRNA and CycT1 (Egloff *et al*, 2006).

The ratio between the active and inactive forms of P-TEFb is tightly regulated to the actual transcriptional demand of the cell (He *et al*, 2006; Zhou and Yik, 2006). Inhibition of global cellular transcription by treatment of cells with actinomycin D (ActD) or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), or irradiation with ultraviolet (UV) light, induces rapid disruption of the 7SK/HEXIM/P-TEFb snRNP to increase the nuclear level of active P-TEFb (Nguyen *et al*, 2001; Yang *et al*, 2001; Michels *et al*, 2003; Yik *et al*, 2003). On the contrary, inhibition of cell growth can shift P-TEFb equilibrium towards the 7SK/HEXIM/P-TEFb snRNP (He *et al*, 2006). In mouse cardiac myocytes, hypertrophic signals facilitate P-TEFb dissociation from the 7SK complex, that eventually results in elevated mRNA and protein synthesis and leads to cardiac hypertrophy (Sano *et al*, 2002).

As compared to P-TEFb, the 7SK snRNA is present in excess within cells (Haaland *et al*, 2003; Michels *et al*, 2003; Byers *et al*, 2005). This raises the possibility that other factors targeting the fraction of 7SK snRNA not associated with HEXIM1 and P-TEFb may also contribute to the

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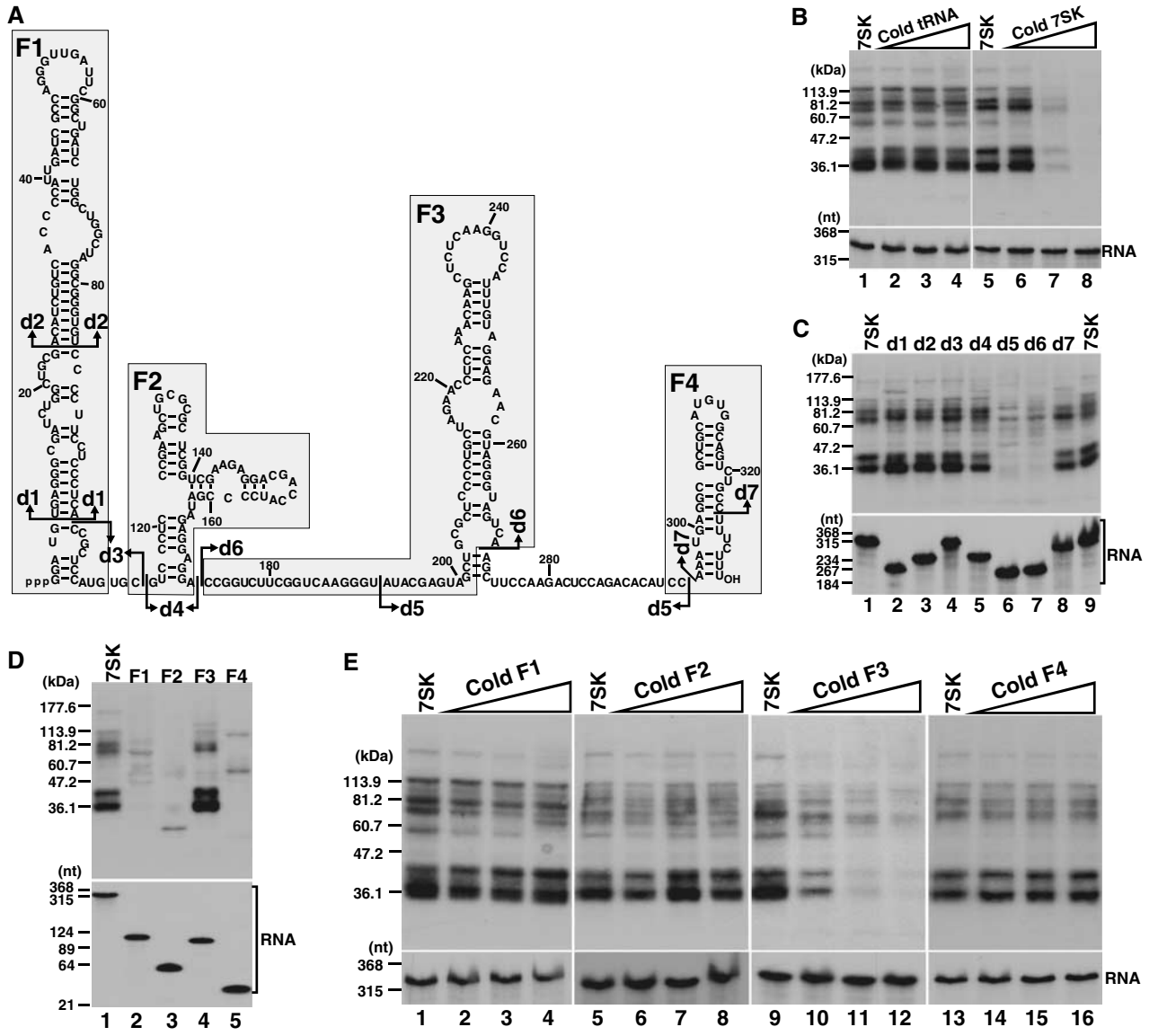


Figure 1 Detection of HeLa proteins interacting with 7SK snRNA *in vitro*. (**A**) Structures of 7SK test RNAs. The secondary structure of human 7SK snRNA has been adopted (Wassarman and Steitz, 1991). The boundaries of internal deletions d1–d7, introduced into the 7SKd1–7SKd7 RNAs, are indicted by arrows. The F1, F2, F3 and F4 fragments of 7SK snRNA are boxed. (**B**) *In vitro* RNA–protein crosslinking. *In vitro* synthesized internally labelled 7SK RNA was incubated with a HeLa nuclear extract. Formation of covalent RNA–protein interactions was induced by irradiation with UV light. After RNase treatment, proteins marked by label transfer were separated on a 12% SDS–polyacrylamide gel and detected by autoradiography. *E. coli* tRNA (lanes 2–4) or cold 7SK snRNA (lanes 6–8) were added in 10- (lanes 2 and 6), 100- (lanes 3 and 7) and 1000-fold (lanes 4 and 8) excess. The stability of 7SK probe RNAs was verified (lower panels). Protein and DNA size markers are indicated. (**C**) *In vitro* crosslinking of internally truncated 7SK RNAs. (**D**) *In vitro* crosslinking of 7SK snRNA fragments. (**E**) *In vitro* crosslinking of 7SK snRNA in the presence of 10- (lanes 2, 6, 10 and 14) 100- (lanes 3, 7, 11 and 15) or 1000-fold (4, 8, 12 and 16) excess of cold F1, F2, F3 or F4 RNAs.

regulation of the nuclear equilibrium of the 7SK/HEXIM/P-TEFb snRNP and free P-TEFb. However, apart from P-TEFb, HEXIM1/2 and the lupus La protein, an RNA-binding protein that functions in stabilization and processing of the 3′ ends of RNAPIII transcribed snRNAs (Wolin and Cedervall, 2002), no additional protein partners have been identified for 7SK snRNA. Here, we demonstrate that a fraction of human 7SK snRNA that is not associated with HEXIM1 and P-TEFb specifically interacts with RNA helicase A, hnRNP A1, A2/B1, R and Q proteins. We provide evidence supporting the idea that dynamic alteration of the nuclear equilibrium between 7SK/HEXIM/P-TEFb and 7SK snRNPs containing the RNA helicase A, hnRNP A1, A2/B1, R and Q proteins plays an fundamental role in controlling the nuclear level of active P-TEFb.

Results

Human 7SK RNA specifically associates with a set of nuclear proteins in vitro

To detect proteins interacting with 7SK snRNA, we performed UV light-induced *in vitro* RNA–protein crosslinking experiments. *In vitro* synthesized, internally labelled 7SK RNA was incubated with a HeLa nuclear extract and irradiated with UV light. The reaction mixture was treated with ribonuclease A and the crosslinked proteins carrying residual radiolabelled

nucleotides originating from the 7SK test RNA were separated by SDS–PAGE and visualized by autoradiography (Figure 1B, lanes 1 and 5). At least six labelled proteins with apparent molecular weights of about 35, 38, 55, 70, 80 and 130 kDa were detected. These proteins interacted with 7SK RNA in the presence of 10-, 100- or 1000-fold excess of nonspecific competitor RNA (Figure 1B, lanes 2–4). However, when increasing amounts of cold 7SK RNA were titrated into the reconstitution reactions, the UV-induced label transfer to proteins was fully abolished, indicating that 7SK RNA forms specific interactions with the labelled proteins (lanes 6–8). The stability of 7SK RNA was verified by PAGE before UV treatment (lower panel).

To define 7SK elements responsible for *in vitro* protein binding, we performed crosslinking experiments with mutant 7SK RNAs carrying nested internal deletions, d1–d7 (Figure 1A). Removal of the d5 and d6 fragments encompassing the third hairpin region of 7SK dramatically reduced the protein binding capacity of 7SKd5 and 7SKd6 RNAs (Figure 1C, lanes 6 and 7). Deletions in the 5′-terminal (d1–d4) or in the 3′-terminal (d7) region of 7SK did not significantly alter the protein binding ability of the truncated RNAs (lanes 2, 3, 4, 5 and 8). Consistent with these observations, a short test RNA, F3 (Figure 1A), representing the third hairpin of 7SK, efficiently interacted with at least three proteins associated with the full-length 7SK RNA (Figure 1D, lane 4). Moreover, cold F3 RNA proved to be an efficient competitor of the assembly of wild-type 7SK RNP (Figure 1E, lanes 10–12). The F1, F2 and F4 RNAs corresponding to the 5′-terminal, the second and the 3′-terminal hairpin regions of 7SK, respectively, failed to efficiently associate with HeLa nuclear proteins (Figure 1D, lanes 2, 3 and 5) or to compete with protein binding to the wild-type 7SK snRNA (Figure 1E). In summary, we conclude that under *in vitro* conditions, the human 7SK snRNA interacts with a set of proteins, which bind predominantly to the third hairpin region of the RNA.

Identification of proteins interacting with 7SK RNA in vitro

To determine the identity of HeLa proteins interacting with 7SK RNA, *in vitro* reconstituted 7SK particles were affinity-selected with a biotinylated 2′-OMe oligoribonucleotide complementary to the human 7SK snRNA from U17 to C33 (Wassarman and Steitz, 1991). Since this oligonucleotide interacted with 7SK sequences essential for HEXIM1 binding, neither HEXIM1 nor P-TEFb was expected to associate with the selected 7SK RNPs (Egloff *et al*, 2006). *In vitro* synthesized 7SK snRNA was annealed to the biotinylated oligonucleotide, immobilized on streptavidin agarose beads and incubated with a HeLa nuclear extract that had been pretreated with micrococcal nuclease. To reduce recovery of proteins nonspecifically bound to the beads, the proteins associated with the immobilized 7SK snRNA were released by ribonuclease treatment. The recovered proteins were resolved on an SDS-polyacrylamide gel (Figure 2, lane 2). As a control, proteins eluted from streptavidin agarose beads coated with biotinylated oligonucleotide, but lacking 7SK RNA, were analysed (lane 1).

The identity of proteins unique to the 7SK-containing beads was determined by MALDI-TOF mass spectrometry (lane 2). The major 7SK-interacting proteins corresponded to the lupus La protein (accession number P05455), RNA helicase A (RHA, Q08211) and unexpectedly, to heterogeneous nuclear ribonucleoprotein (hnRNP) A1 (P09651), A2/B1 (P22627), R (O43390) and Q (O60506). Notably, the apparent molecular masses of hnRNP A1, A2/B1 (36–40 kDa), La (55 kDa), hnRNP Q (70 kDa), hnRNP R (80 kDa) and RHA (140 kDa) showed a great correlation with the estimated masses of HeLa proteins detected by *in vitro* RNA–protein crosslinking (Figure 1). The minor bands indicated by asterisks represented proteins, which showed no significant *in vivo* interaction with 7SK snRNA in co-immunoprecipitation (co-IP) studies (data not shown, see legend to Figure 2). Therefore, the significance of these proteins remains uncertain.

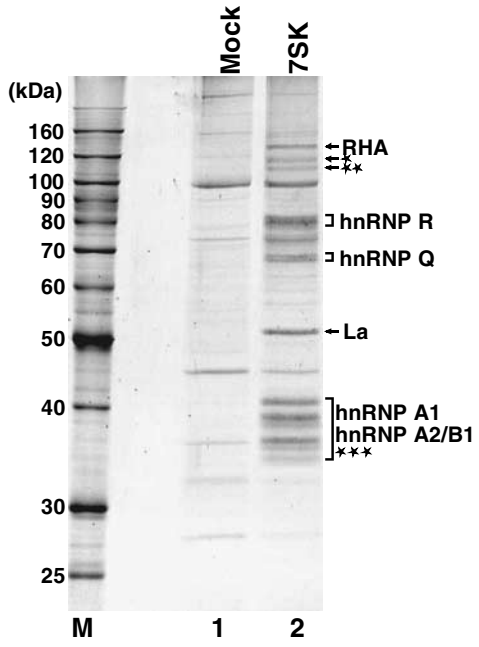


Figure 2 Identification of HeLa proteins associated with 7SK RNA. *In vitro* assembled 7SK RNP particles were affinity-selected with a biotinylated antisense oligoribonucleotide immobilized on streptavidin agarose beads. Proteins associated with beads in the absence (mock) or presence of 7SK RNA were analysed by SDS–PAGE. Proteins specific for 7SK-containing beads were isolated and subjected to mass spectrometry. Minor proteins bands indicated by asterisks correspond to *hnRNP U, **DRBP76 and ***hnRNP C1. IP of HeLa hnRNP C1 or transiently expressed HA-tagged DRBP76 and hnRNP U proteins failed to recover significant amounts of 7SK snRNA (data not shown).

In vivo association of 7SK snRNA with RHA, hnRNP A1, A2/B1, R and Q

We tested whether the newly identified *in vitro* binding partners of 7SK RNA are integral components of the human 7SK snRNP in living cells. In the first approach, a 5′-end-tagged 7SK RNA carrying a binding motif for bacteriophage MS2 coat protein was transiently expressed in HeLa cells (Figure 3A, lane 1). The ectopically expressed MS2-7SK RNA and the endogenous 7SK snRNA, but not U2 and MRP snRNAs, co-immunoprecipitated with HeLa CycT1 and transiently expressed HA-tagged HEXIM1, indicating that the MS2-7SK RNA was incorporated into functional snRNPs (lanes 2 and 3). The expressed MS2-7SK snRNA was affinity selected with a recombinant MS2 coat protein that had been fused to maltose-binding protein (MBP) and immobilized on

amylose resin (Zhou *et al*, 2002). After elution with maltose, the recovered RNA was terminally labelled and analysed on a sequencing gel (Figure 3B, lane 3). Autoradiography detected one major RNA band that corresponded to the MS2-7SK RNA. The endogenous 7SK snRNA did not co-purify with MS2-7SK RNA, indicating that HeLa 7SK snRNP contains only one 7SK molecule (Li *et al*, 2005). No RNA was recovered by mock purification from non-transfected HeLa cells (lane 2).

Proteins co-selected with MS2-7SK RNA were assayed by immunoblot analysis with antibodies against hnRNP A1, A2 and Q (Figure 3C). Human cells express three alternatively spliced versions of hnRNP Q, namely Q1, Q2 and Q3, which share more than 80% identity with hnRNP R (Mourelatos *et al*, 2001). Therefore, the antibody against hnRNP Q also recognized hnRNP R. Likewise, the anti-hnRNP A2 antibody also highlighted hnRNP B1, since the amino-acid sequences of these proteins are identical, except for an additional 12 amino acids inserted at the N-terminus of hnRNP B1. Due to the lack of a specific antibody, *in vivo* interaction of RHA with MS2-7SK RNA was tested by coexpression of a FLAG-tagged RHA (FL-RHA). The transiently expressed FL-RHA protein, as well as HeLa hnRNP A1, A2, B1, R and the Q1 and Q3 isoforms of hnRNP Q, but not Q2, co-purified with MS2-7SK (lane 3). None of these proteins was detectable upon mock purification of MS2-7SK from non-transfected cells (lane 2). As expected, HEXIM1 co-purified with MS2-7SK, but an unrelated protein, the mitogen-activated protein kinase 2 (MAPK2), was not detectable in the MS2-7SK purification.

Next, we assayed whether HeLa endogenous 7SK snRNA interacts with RHA, hnRNP A1, A2/B1, R and Q proteins in living cells. With the exception of anti-A1 antibody, the commercially available antibodies against hnRNPs poorly performed in IP reactions (data not shown). Therefore, haemagglutinin (HA), FLAG (FL) or paramyxovirus SV5 (V5) epitope-tagged hnRNP R (HA-R), Q1 (HA-Q1), Q2 (HA-Q2), A2 (A2-V5), B1 (B1-V5) and RHA (FL-RHA) proteins were transiently expressed in HeLa cells. Western blot analysis revealed that the ectopically expressed tagged proteins accumulated at levels comparable to the endogenous proteins (Figure 3D). After IP with appropriate antibodies, the recovery of each target protein was confirmed by Western blotting and co-precipitation of 7SK snRNA was tested by Northern blotting (Figure 3E). Probing the blots with a 7SK-specific probe demonstrated that the HeLa hnRNP A1 and the transiently expressed HA-R, HA-Q1, A2-V5, B1-V5 and FL-RHA proteins interacted with 7SK snRNA, but they showed no significant interaction with U2, U6 and/or MRP snRNAs. As already indicated by affinity selection of the MS2-7SK snRNP (Figure 3C), IP of HA-Q2 failed to pull down 7SK snRNA, demonstrating that Q2 that lacks the N-terminal portion of its second RNA recognition motif (RRM) cannot interact with 7SK snRNA.

Finally, we also performed co-IP experiments from extracts prepared from formaldehyde crosslinked HeLa cells under stringent conditions (Niranjanakumari *et al*, 2002; Figure 3F). Although the anti-HA and anti-FLAG antibodies failed to retain their binding capacity under the harsh wash conditions applied to destroy non-covalent RNA–protein interactions, the anti-A1 and anti-V5 antibodies pulled down hnRNP A1, A2-V5 and B1-V5 proteins. RNase A/T1 protection analysis demonstrated that the 7SK snRNA, but not U2, was efficiently crosslinked *in vivo* to hnRNP A1 and A2-VA and B1-V5 proteins. Based on these above results, we concluded that human RHA, hnRNP A1, A2, B1, R, Q1 and most probably Q3 specifically interact with 7SK snRNA in living cells.

7SK elements directing in vivo binding of the novel 7SK snRNP proteins

To outline 7SK regions directing *in vivo* recruitment of the newly identified 7SK snRNP proteins, we investigated the interaction of transiently expressed, internally truncated 7SK RNAs (Figure 1A) with HeLa hnRNP A1 and transiently expressed HA-R, HA-Q1 and A2-V5 proteins (Figure 4A). After IP, RNAs recovered from the supernatants (lanes S) and pellets (lanes P) were analysed by Northern blotting with 7SK-specific probes. The hnRNP A1, A2-V5, HA-R and HA-Q1 proteins, although bound to the endogenous wild-type 7SK snRNA, failed to interact with the truncated 7SKd5 and 7SKd6 RNAs, demonstrating that the third, internal hairpin of 7SK snRNA plays a central role in 7SK snRNP assembly.

Binding of HA-R and HA-Q1 was also abolished by removal of the 5′-terminal hairpin of 7SKd1, showing that *in vivo* recruitment of hnRNP R and Q1 requires binding elements both in the 5′-terminal and the third hairpin of 7SK snRNA. Apart from the d5 and d6 deletions, other internal truncations failed to inhibit the *in vivo* binding of hnRNP A1 and A2-V5, indicating that all the elements required for efficient and specific binding of hnRNP A1 and A2 are contained in the third hairpin of 7SK. Indeed, the transiently expressed 7SK-3HP RNA that represented the third hairpin region of 7SK from G196 to C277 specifically interacted with both hnRNP A1 and A2-V5 (Figure 4B). For binding of RHA, we failed to identify fully indispensable 7SK elements, although the lack of the 5′-hairpin considerably reduced interaction of 7SKd1 with transiently expressed FL-RHA (data not shown).

Human 7SK snRNA resides in at least three distinct snRNP particles

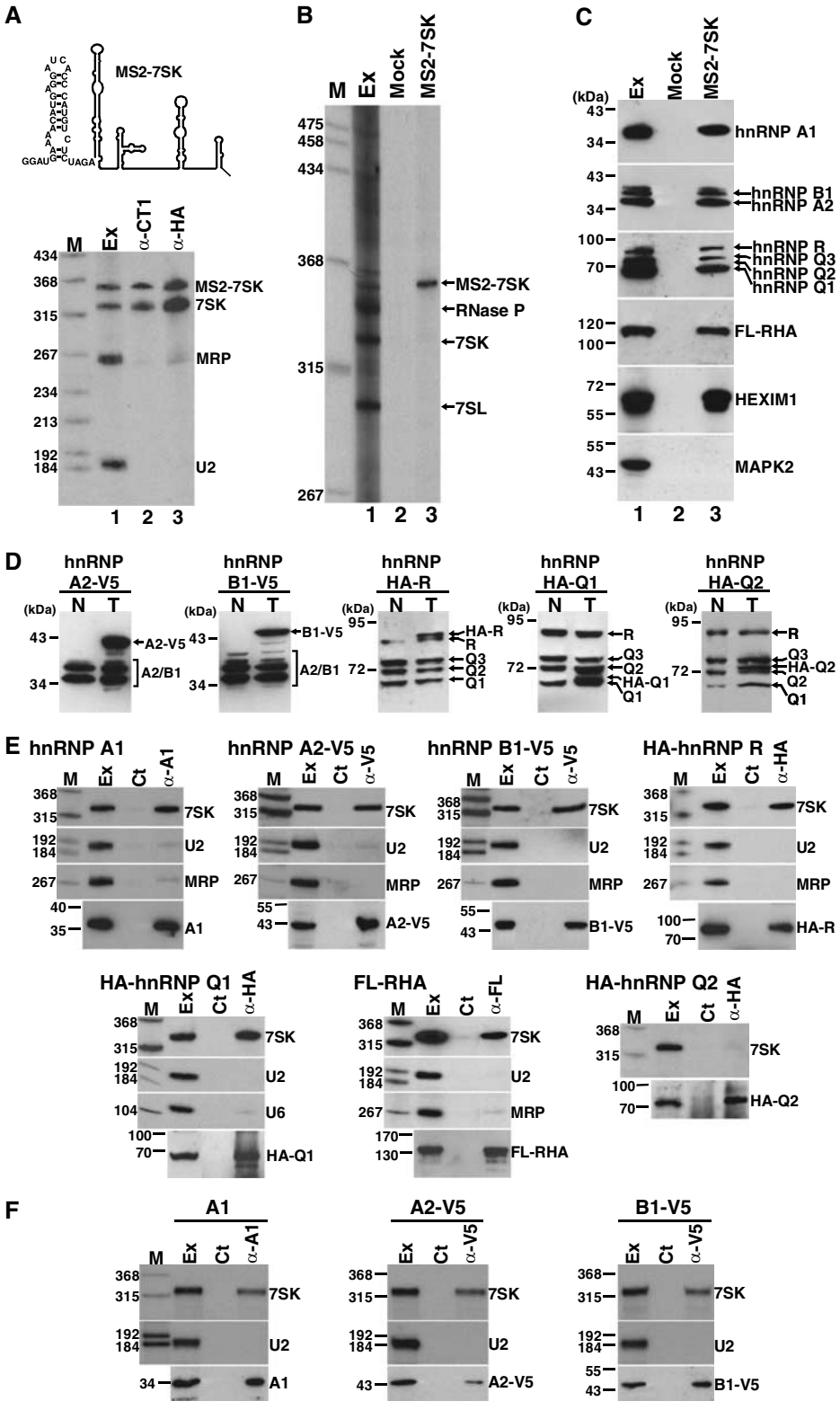
Next we investigated whether the novel 7SK snRNP proteins associate with the 7SK/HEXIM1/P-TEFb snRNP. From a HeLa cell extract, P-TEFb was immunoprecipitated with an anti-CycT1 antibody and the co-precipitated proteins were analysed by Western blotting (Figure 5A). As expected, the anti-CycT1 antibody pulled down both HEXIM1 and 7SK snRNA (lane 3), but HeLa hnRNP A1, A2/B1, R and Q and transiently expressed FL-RHA were not detectable in the pellet (lane 3). These results were confirmed by reciprocal co-IP experiments, in which precipitation of HeLa hnRNP A1 and transiently expressed HA-R and FL-RHA failed to pull down CycT1 (Figure 5B, lanes 3, 6 and 9). Thus, we conclude that none of the newly identified 7SK RNP proteins interact with 7SK/HEXIM1/P-TEFb.

Interestingly, upon IP of hnRNP A1, neither hnRNP R nor hnRNP Q proteins was detected in the pellet (Figure 5B, lane 3). On the other hand, IP of transiently expressed HA-R failed to pull down HeLa hnRNP A1 and A2/B1 and transiently expressed FL-RHA (lane 6). These results demonstrate that besides the well-characterized 7SK/HEXIM/P-TEFb complex, human 7SK snRNA forms at least two additional RNP particles, which either contain or lack hnRNP A1, R and Q proteins. Unfortunately, given that hnRNP proteins are known to form huge multiprotein complexes, our observation that some of the novel 7SK snRNP proteins, for example, hnRNP A1 and A2/B1, as well as hnRNP R and Q, are

associated in the nucleus (data not shown), cannot be positively interpreted concerning the protein composition of 7SK snRNPs.

Transcription inhibition induces dynamic remodelling of human 7SK snRNPs

The experiments presented thus far demonstrate that hnRNP A1, A2/B1, R, Q and RHA form 7SK snRNP particles distinct from the 7SK/HEXIM/P-TEFb snRNP. Previous studies revealed that inhibition of global cellular transcription by ActD or DRB treatment increases the nuclear level of active P-TEFb by inducing the disassembly of 7SK/HEXIM/P-TEFb (Nguyen *et al*, 2001; Yang *et al*, 2001; Michels *et al*, 2003; Yik *et al*, 2003; Byers *et al*, 2005). We tested whether upon stress-induced disruption of 7SK/HEXIM/P-TEFb, the dissociated 7SK snRNAs form snRNPs with the newly discovered 7SK



snRNP proteins (Figure 6). As reported before, 1 h ActD treatment reduced the interaction of HeLa 7SK snRNA with CycT1 by 80% as compared to control cells (Figure 6A, panel CycT1, lanes 4 and 5). ActD treatment had no effect on the accumulation of 7SK snRNA, and therefore RNA degradation cannot account for the decreased association of CycT1 with 7SK snRNA (lanes 1 and 2).

Next, we compared the interactions of 7SK snRNA with hnRNP A1 and transiently expressed HA-R, HA-Q1, A2-V5 and FL-RHA proteins in ActD-treated and non-treated cells (Figure 6A, lanes 4 and 5). In marked contrast to CycT1, hnRNP A1, HA-R, HA-Q1, A2-V5 and FL-RHA showed a largely increased (from 300 to 550%) association with 7SK snRNA in ActD-treated cells (see also Figure 6B). Similar results were obtained when HeLa cells were treated with DRB (Figure 6C). An hour after DRB administration, the interaction of 7SK snRNA with CycT1 was largely diminished (lane 2), but on the other hand, 7SK showed an augmented association with hnRNP A1, A2-V5, HA-R, HA-Q1 and FL-RHA proteins (lanes 5, 8, 11, 14 and 17). Importantly, 1 h after removal of DRB, the original nuclear level of 7SK/HEXIM/P-TEFb was restored (lane 3), and concomitantly, the amounts of 7SK snRNA associated with hnRNP A1, A2-V5, HA-R, HA-Q1 and FL-RHA proteins were reduced to the levels observed in non-treated cells (lanes 3, 6, 9, 12, 15 and 18). These results indicate that the increase of the nuclear level of active P-TEFb in response to transcription inhibition is supported by a rapid and reversible conversion of the 7SK/HEXIM/P-TEFb snRNP into the newly discovered '7SK/hnRNP' particles. We propose that there exists a dynamic functional equilibrium between the 7SK/HEXIM1/P-TEFb-negative and the novel '7SK/hnRNP'-positive transcriptional regulatory complexes that eventually, determines the nuclear level of active P-TEFb.

The third hairpin of 7SK directing hnRNP A1, A2, R and Q binding is essential for in vivo disassembly of 7SK/HEXIM/P-TEFb

To learn more about the function of the novel 7SK snRNP proteins, we investigated the dynamic interaction of P-TEFb with the internally truncated 7SKd5 and 7SKd6 RNAs. Due to the lack of the third hairpin, 7SKd5 and 7SKd6 could not interact with hnRNP A1, A2, R and Q (Figure 4), but they were expected to bind both HEXIM1 and P-TEFb (Egloff *et al*, 2006). HeLa cells transiently expressing 7SKd5, 7SKd6 or a control RNA, 7SKd4, that interacts with all known 7SK snRNP proteins, were treated with ActD and interaction of the mutant 7SK RNAs with P-TEFb was investigated by IP with anti-CycT1 antibody (Figure 7A). As evidenced by Northern blot analysis of total RNAs obtained from cell extracts, ActD treatment had no significant effect on the accumulation of 7SKd4, 7SKd5 and 7SKd6 (lanes 1–6). The 7SKd4, 7SKd5 and 7SKd6 RNAs, like the endogenous 7SK, co-immunoprecipitated with CycT1, demonstrating that they interact with HEXIM1 and P-TEFb (lanes 7, 9 and 11). ActD treatment diminished the association of CycT1 with the endogenous 7SK and the transiently expressed 7SKd4 snRNAs (lanes 8, 10 and 12), but it failed to reduce the interaction of CycT1 with 7SKd5 and 7SKd6 (lanes 10 and 12). This demonstrates that the 7SKd5/HEXIM/P-TEFb and 7SKd6/HEXIM/P-TEFb snRNPs harbouring mutant 7SK RNAs lacking the capacity to recruit hnRNP A1, A2, R and Q are resistant to ActD-induced disassembly. These results indicate that binding of the newly discovered 7SK snRNP proteins is essential for stress-induced disassembly of 7SK/HEXIM1/P-TEFb, and consequently, for regulation of the nuclear level of active P-TEFb.

Discussion

The human 7SK snRNA functions as a key regulator of RNAPII transcription elongation by controlling the CTD kinase activity of P-TEFb. The 7SK snRNA sequesters P-TEFb into the kinase-inactive 7SK/HEXIM/P-TEFb snRNP and thus, regulates the fraction of P-TEFb available for CTD phosphorylation. In HeLa cells, only a subfraction of the nuclear pool of 7SK snRNA has been found in the 7SK/HEXIM/P-TEFb snRNP, leading to the postulation that the rest of 7SK interacts with other, not yet identified snRNP proteins (Michels *et al*, 2003; Yik *et al*, 2003). In this study, development of an *in vitro* 7SK snRNP reconstitution system, followed by affinity purification of 7SK particles, led to the identification of RHA, hnRNP A1, A2/B1, R and two variants of hnRNP Q, hnRNP Q1 and Q3, as novel 7SK-interacting proteins that specifically associate with human 7SK snRNA both in the test tube and in living cells (Figures 1, 2 and 3).

The novel 7SK snRNP proteins seem to directly interact with 7SK snRNA and they carry conserved structural domains devoted to RNA binding. The closely related hnRNP A1 and A2/B1 proteins contain two, while hnRNP R and Q possess

Figure 3 *In vivo* association of 7SK snRNA with RHA and hnRNP A1, A2/B1, R and Q proteins. (**A**) Transient expression of MS2-7SK RNA. A schematic representation of MS2-7SK RNA with highlighted MS2-binding sequences is shown. From a sonic extract of HeLa cells expressing the MS2-7SK RNA and HA-tagged HEXIM1, snRNPs were immunoprecipitated with anti-CycT1 (α-CT1) and anti-HA (α-HA) antibodies. RNAs extracted from the pellets were fractionated on a 6% sequencing gel, electroblotted onto a nylon membrane and probed with a mixture of labelled oligonucleotides specific for the human 7SK, U2 and MRP snRNAs. Lane Ex, RNAs from HeLa cell extract; lane M, size markers. (**B**) Affinity selection of MS2-7SK RNA with a recombinant MS2-MBP fusion protein. RNAs affinity-selected from HeLa cell extracts not expressing (mock) or expressing the MS2-7SK RNA were terminally labelled with cytidine 3′,5′-bis(phosphate) (pCp) and T4 RNA ligase and analysed on a 6% sequencing gel. Lane Ex, RNAs from HeLa cell extract expressing MS2-7SK. (**C**) Proteins associated with transiently expressed MS2-7SK RNA. Proteins co-purified with MS2-7SK RNA or mock-purified from a cell extract not expressing MS2-7SK (mock) were immunoblotted with antibodies as indicated on the right. Lane Ex, cell extract. Protein size markers are indicated. (**D**) Transient expression of epitope-tagged hnRNP proteins. Proteins extracted from HeLa cells either transfected (T) or not transfected (N) with expression vectors for hnRNP A2-V5, B1-V5, HA-R HA-Q1 and HA-Q2 were analysed by Western blotting with specific antibodies. (**E**) Proteins interacting with HeLa 7SK snRNA. RNAs co-immunoprecipitated with HeLa hnRNP A1 or with transiently expressed hnRNP A2-V5 (A2-V5), hnRNP B1-V5 (B1-V5), HA-hnRNP R (HA-R), HA-hnRNP Q1 (HA-Q1), HA-hnRNP Q2 (HA-Q2) and FL-RHA proteins were tested by Northern blotting. RNAs recovered from cell extracts (Ex) or immunoprecipitated with non-specific IgG (Ct) were also assayed. IP of proteins was verified by Western blot analysis (lower panels). (**F**) *In vivo* RNP-IP. Transfected (A2-V5, B1-V5) or non-transfected (A1) HeLa cells were cross-linked with formaldehyde. After extract preparation, hnRNP A1, A2-V5 and B1-V5 were immunoprecipitated under stringent conditions. Co-precipitation of 7SK and U2 snRNA was tested by RNase A/T1 mapping. RNAs extracted from one fiftieth of the extracts (Ex) or mock-precipitated with nonspecific IgG (Ct) were also mapped. IP of proteins was verified by Western blotting (lower panels).

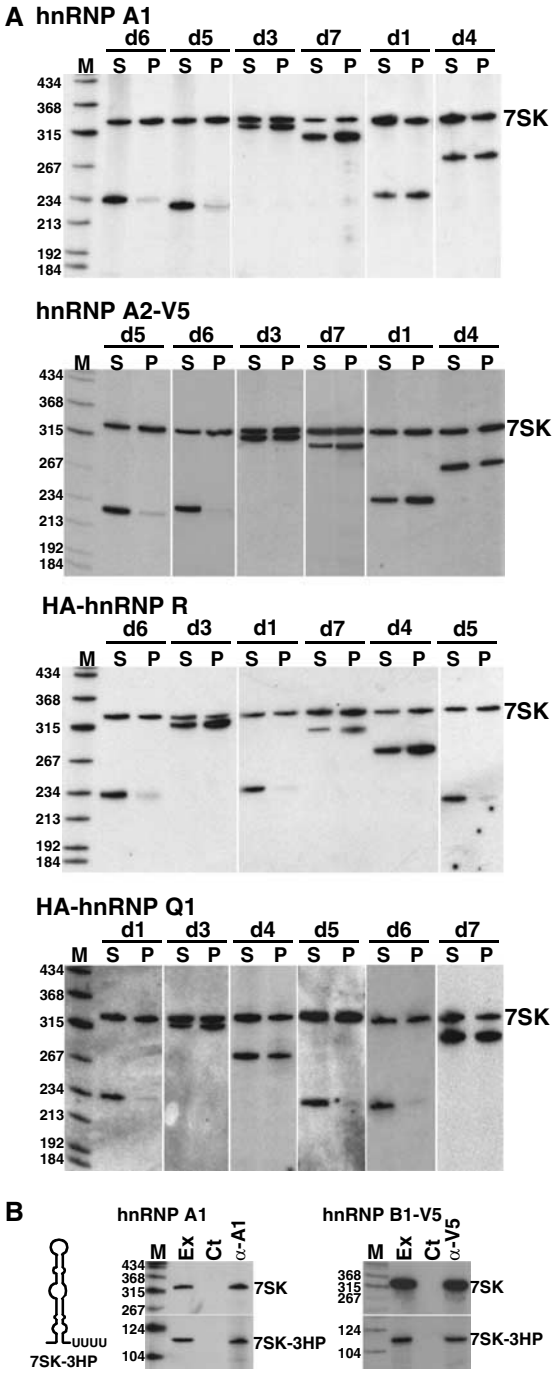


Figure 4 7SK elements directing *in vivo* binding of hnRNP A1, A2, R and Q1. (**A**) Analysis of the protein binding capacity of internally truncated 7SK RNAs. Mutant 7SK RNAs, indicated above the lanes, were transiently expressed in HeLa cells also expressing epitope-tagged hnRNP A2-V5, HA-hnRNP R or HA-hnRNP Q1 proteins. Cellular sonic extracts were prepared and 7SK snRNPs were immunoprecipitated with anti-hnRNP A1, anti-HA or anti-V5 antibodies. RNAs extracted from the pellets (P) and supernatants (S) were analysed by Northern blotting with 7SK-specific oligonucleotide probes. Endogenous 7SK snRNA is shown. (**B**) 7SK-3HP RNA corresponding to the G196-C277 region of human 7SK snRNA interacts with hnRNP A1 and A2. 7SK-3HP RNA and hnRNP A2-V5 protein were transiently coexpressed in HeLa cells. RNAs purified from cell extract (Ex) or co-immunoprecipitated with hnRNP A1 (α-A1) or A2-V5 (α-V5) were subjected to Northern blot analysis. Lanes Ct, control IP with nonspecific IgG.

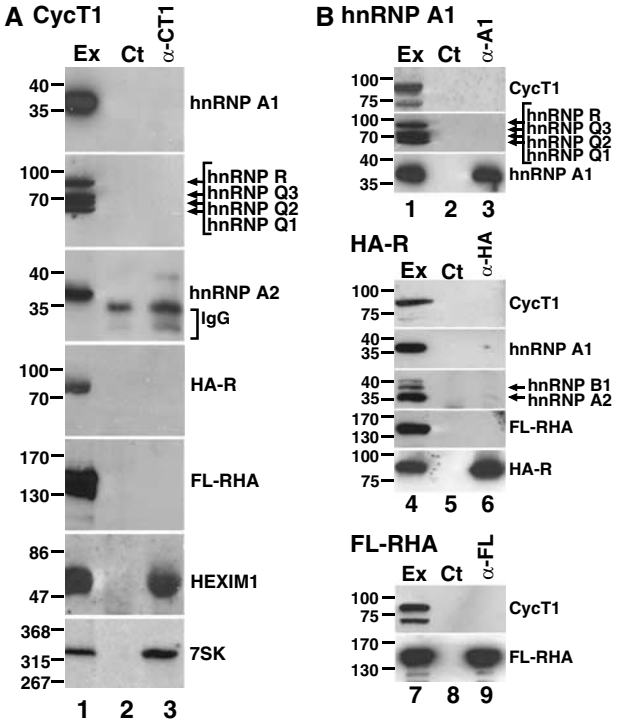


Figure 5 HeLa 7SK snRNA exists in multiple snRNPs. (**A**) Proteins associated with 7SK/HEXIM/P-TEFb snRNP. Proteins immunoprecipitated from a HeLa cell extract with an anti-CycT1 antibody (α-CT1) or with nonspecific IgG (Ct) were separated on a 12% SDS-polyacrylamide gel, electroblotted onto a nitrocellulose membrane and stained with antibodies indicated on the right. Lane Ex, cell extract. (**B**) HeLa hnRNP A1, hnRNP R and RHA do not associate with P-TEFb. Proteins co-immunoprecipitated with HeLa hnRNP A1 (α-A1) and transiently expressed HA-R (α-HA) and FL-RHA (α-FL) were characterized by Western blotting with antibodies indicated on the right.

three, RRMs (also called RNP) domains, and they all carry RGG (Arg–Gly–Gly) repeat boxes (Krecic and Swanson, 1999; Dreyfuss *et al*, 2002). RHA has two double-stranded RNA-binding motifs (DRBM) and also carries GY-rich sequence repeats highly reminiscent of the RGG boxes of hnRNP A1, A2/B1, R and Q (Zhang and Grosse, 1997). Numerous RNA-binding proteins involved in mRNA and rRNA metabolism contain RRM domains and RGG boxes, pointing to the enormous flexibility of these structural motifs in recognition of a broad range of RNA substrates. *In vitro* and *in vivo* binding experiments demonstrated that the novel 7SK snRNP proteins recognize particular structural elements of the 7SK snRNA (Figures 1 and 4). The hnRNP A1 and A2 proteins bind specifically and exclusively to the third hairpin of 7SK. It is noteworthy that this region of 7SK carries a putative hnRNP A1-binding sequence, 260-UAGGGU-265, identical to the experimentally defined optimal binding motif for hnRNP A1 (Burd and Dreyfuss, 1994). Efficient binding of hnRNP R and Q1 requires docking elements both in the 5′-terminal and third hairpin of 7SK snRNA. Thus far, no 7SK regions, which are absolutely essential for RHA binding have been identified. This might indicate that RHA has multiple binding sites in 7SK snRNA and/or protein–protein interactions may also contribute to the tethering of RHA to 7SK snRNP.

The third hairpin region that is fundamental for binding of hnRNP A1, A2, R and Q shows a strong sequence conservation in vertebrate 7SK snRNAs, pointing to the functional

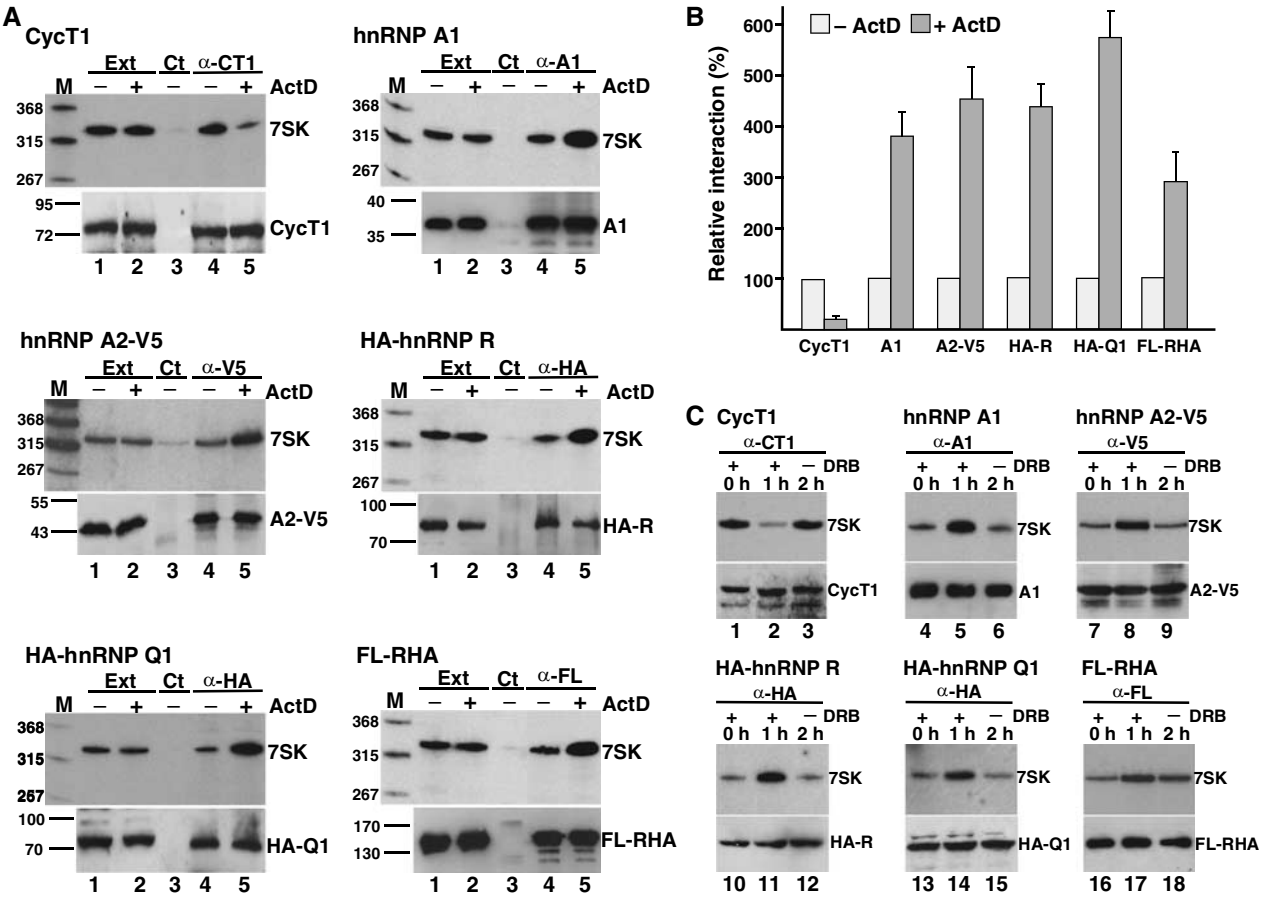


Figure 6 Dynamic remodelling of HeLa 7SK snRNP. (**A**) ActD treatment of HeLa cells increases the nuclear levels of 7SK snRNPs containing RHA, hnRNP A1, A2, R and Q. Epitope-tagged hnRNP A2-V5, HA-hnRNP R, HA-hnRNP Q1 and FL-RHA proteins were transiently expressed in HeLa cells. RNAs phenol-extracted (Ext) or immunoprecipitated (antibodies are indicated above the lanes) from extracts prepared from ActD-treated (+) or non-treated (−) cells were analysed by Northern blotting (upper panels). The efficacy of IPs was monitored by Western blot analysis (lower panels). Lanes Ct, control IPs with nonspecific IgG; lanes M, size markers. (**B**) Relative association of HeLa 7SK snRNA with 7SK snRNP proteins in ActD-treated HeLa cells. The intensities of 7SK snRNAs detected by Northern hybridization were quantified by phosphorimager. The relative levels of 7SK were normalized to the snRNP proteins recovered by IP. The levels of 7SK RNAs immunoprecipitated from non-treated control cells were set as 100%. Standard deviations are indicated. (**C**) Dynamics of 7SK snRNP in DRB-treated HeLa cells. HeLa hnRNP A1 and transiently expressed hnRNP A2-V5, HA-hnRNP R, HA-hnRNP Q1 and FL-RHA were immunoprecipitated, and association of 7SK snRNA was monitored by Northern blot analysis. Extracts for IP were prepared immediately after administration (0 h), 1 h after administration (1 h) and 1 h after removal of DRB (2 h). The efficiency of IP was confirmed by Western blot analysis (lower panels).

importance of the interacting proteins (Egloff *et al*, 2006). Thus far, the only known molecular function of 7SK snRNA is the inhibition of the CTD kinase activity of P-TEFb. Our co-IP experiments, however, demonstrated that none of the new 7SK snRNP proteins associate with the 7SK/HEXIM1/P-TEFb snRNP, indicating that they are not required for the 7SK-mediated inactivation of P-TEFb (Figure 5). Consistent with this, the 7SKd5 and 7SKd6 RNAs that are incapable of binding hnRNP A1, A2, R and Q can recruit both HEXIM1 and P-TEFb and can inhibit transcription (Figure 7 and our unpublished data).

Our data strongly support the idea that instead of inactivating the kinase activity of P-TEFb, the novel 7SK snRNP proteins contribute to the controlling of the steady-state level of the 7SK/HEXIM/P-TEFb snRNP, and by this means, they modulate the nuclear level of free P-TEFb. We have shown that transcription inhibition by ActD or DRB treatment induces the disassembly of 7SK/HEXIM1/P-TEFb, and at the same time, increases the level of 7SK snRNPs containing RHA, hnRNP A1, A2/B1, R and Q (Figure 6). Importantly, elimination of the transcription inhibitors restores the original levels of both 7SK/HEXIM/P-TEFb and 7SK/hnRNP complexes, indicating that 7SK snRNPs undergo dynamic and reversible remodelling in response to transcription inhibition. We propose that there is a dynamic functional equilibrium between the 7SK/HEXIM/P-TEFb-negative transcriptional regulatory snRNP and newly identified 7SK/hnRNP complexes. The nuclear ratio of the 7SK/HEXIM/P-TEFb and 7SK/hnRNP complexes is tightly regulated according to the transcriptional need of the cell (Figure 8). For instance, disruption of global cellular transcription shifts the 7SK snRNP equilibrium towards the 7SK/hnRNP complexes and consequently, increases nuclear level of active P-TEFb. An important question is whether during stress-induced remodelling of 7SK snRNPs, binding of the novel 7SK snRNP proteins, serves as a cause or as a consequence of 7SK/HEXIM/P-TEFb disassembly. Our finding that 7SKd5/HEXIM/P-TEFb and 7SKd6/HEXIM/P-TEFb particles containing

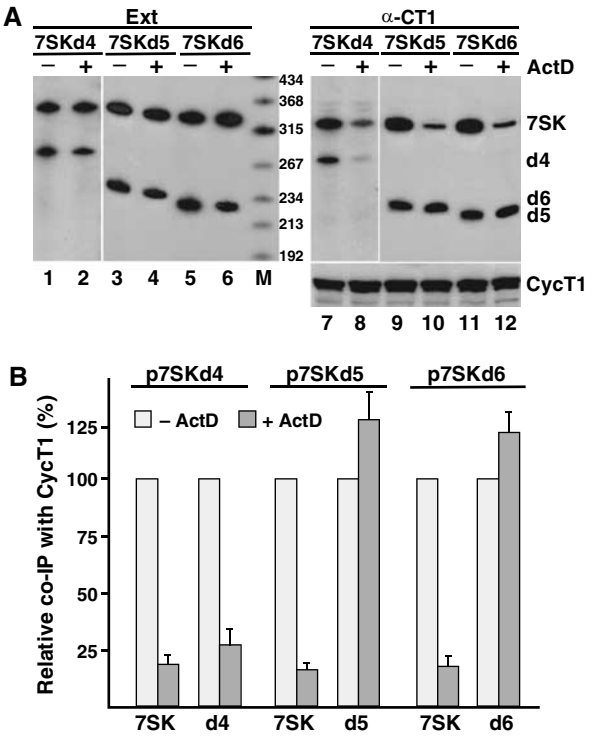


Figure 7 The third hairpin of 7SK is required for ActD-induced disassembly of 7SK/HEXIM/P-TEFb. (**A**) 7SKd5 and 7SKd6 RNAs stably associate with P-TEFb. HeLa cells expressing 7SKd4, 7SKd5 or 7SKd6 RNAs were treated (+) or non-treated (−) with ActD for 1 h before extract preparation. RNAs phenol-extracted (Ext) or immunoprecipitated with an anti-CycT1 (α-CT1) antibody were analysed by Northern blotting. IP of CycT1 was verified by Western blot analysis (lower panel). (**B**) Relative association of 7SK, 7SKd4, 7SKd5 and 7SKd6 RNAs with CycT1 in ActD-treated and control cells. The relative levels of 7SK, 7SKd4, 7SKd5 and 7SKd6 RNAs immunoprecipitated from control cells were set as 100%. The expression constructs (p7SKd4, p7SKd5 and p7SKd6) used for transfection of HeLa cells are indicated.

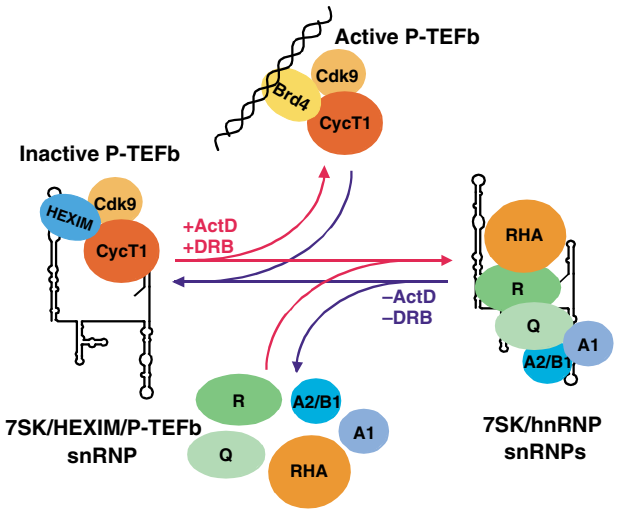


Figure 8 A model for regulation of the nuclear level of active P-TEFb by dynamic and reversible remodelling of 7SK snRNPs. In exponentially growing HeLa cells, about 50% of P-TEFb is sequestered into the 7SK/HEXIM1/P-TEFb snRNP, while the other half associates with the bromodomain protein 4 (Brd4) that likely recruits active P-TEFb to chromatin templates. Transcription inhibition by ActD or DRB treatment induces dissociation of P-TEFb and HEXIM1 from the 7SK snRNA and at the same time, facilitates binding of RHA, hnRNP A1, A2/B1, R and Q proteins. Please note that two copies of HEXIM1 and P-TEFb interact with one 7SK molecule in the 7SK/HEXIM1/P-TEFb snRNP, and that at least two different 7SK/hnRNP particles with not yet fully clarified protein composition are formed upon transcription inhibition.

mutant 7SK RNAs incompetent in hnRNP A1, A2, R and Q binding are resistant to stress-induced disassembly suggests that binding of the novel 7SK snRNP proteins is a prerequisite for, rather than a consequence, of 7SK/HEXIM1/P-TEFb disassembly (Figure 7).

The hnRNP proteins have long been known to function in packaging of the newly synthesised pre-mRNAs, alternative splicing, nucleo-cytoplasmic transport, stability and translation of mRNAs (Krecic and Swanson, 1999; Dreyfuss *et al*, 2002). The finding that hnRNP A1, A2/B1, R and Q proteins are involved in regulation of RNAPII transcription elongation indicates that hnRNP proteins play a more complex role in mRNA biogenesis than anticipated before. An intriguing question is whether the newly identified 7SK/hnRNP snRNPs, besides controlling the level of active P-TEFb, possess additional nuclear function(s). Notably, hnRNP R, hnRNP Q and RHA can specifically associate with RNAPII (Nakajima *et al*, 1997; Carty and Greenleaf, 2002), although it is unclear whether they recruit 7SK snRNA to the RNAPII transcriptional machinery.

The notion that control of the nuclear level of active P-TEFb is supported by dynamic remodelling of the 7SK snRNP raises several questions. It is unclear how the 7SK/HEXIM1/P-TEFb snRNP and the newly described 7SK/hnRNP complexes are formed in a mutually exclusive manner, since these proteins recognize distinct elements of the 7SK snRNA. In principle, it is possible that binding of hnRNP R and Q to the 5′-hairpin of 7SK hinders the interaction of HEXIM1 with 7SK snRNA. But hnRNP A1 and A2 would be expected to associate with the third hairpin of 7SK snRNA even when it is incorporated into the 7SK/HEXIM1/P-TEFb snRNP. Of course, it is quite possible that besides the currently known proteins, 7SK snRNPs carry additional, not yet identified snRNP protein(s) with decisive roles in 7SK snRNP remodelling. Another formal possibility is that remodelling of 7SK snRNP is accompanied or is even driven by conformational changes in the three-dimensional structure of 7SK snRNA, that could eventually define the actual range of 7SK snRNP proteins to be recruited. It is tempting to speculate that RHA might function in the dynamic structural reorganization of 7SK snRNA. Finally, it is also possible that dynamic remodelling of the 7SK snRNP is supported by *trans*-acting factors, which temporarily interact with 7SK snRNPs. Along this line, it is important to mention that RHA, hnRNP R and Q have been reported to specifically interact with the survival of motor neurons (SMN) protein that, as an integral component of the SMN complex, functions in the assembly of various classes of nuclear snRNPs (Campbell *et al*, 2000; Mourelatos *et al*, 2001; Pellizzoni *et al*, 2001; Rossoll *et al*, 2002). Thus, understanding of the molecular mechanism underlying the structural dynamics of human 7SK snRNP will be a challenging task for the future.

Materials and methods

Unless stated otherwise, all manipulations were performed according to standard laboratory protocols (Sambrook *et al*, 1989).

Modified and unmodified oligonucleotides were synthesized by Eurogentech.

Expression constructs and transient expression

Construction of p7SK and p7SKd1 to p7SKd7 expression plasmids has been described (Egloff *et al*, 2006). The pMS2-7SK expression construct that carried a human 7SK snRNA gene in which the 5′-end of the 7SK coding region was fused to a MS2 coat protein binding motif was generated by PCR amplification using p7SK as a template. Full-length cDNAs encoding human hnRNP Q1, Q2 and R proteins carrying N-terminal HA tags were amplified by RT–PCR from HeLa total RNA and cloned into the pcDNA3 vector (Invitrogen). Amplified full-length cDNAs of hnRNP A2 and B1 were inserted into the pEF1/V5-His B vector (Invitrogen). To express FLAG-tagged human RHA protein, we utilized the pCDNA3-fhis-RHA expression plasmid (Zhou *et al*, 2003). The identity of each construct was verified by sequence analysis. Human HeLa cells were grown in Dulbecco's modified Eagle medium supplemented with 10% foetal calf serum (Invitrogen).

Protein and RNA analysis

Preparation of cell extract, IP and Western blot analysis of proteins, Northern blot analysis and RNase A/T1 mapping of RNAs have been described (Egloff *et al*, 2006). *In vivo* RNP-IP was performed according to Niranjanakumari *et al* (2002). Anti-HA (12CA5, Roche), anti-FLAG (M2, Sigma), anti-hnRNP A1 (4B10, Abcam), anti-hnRNP A2/B1 (DP3B3, Sigma), anti-hnRNP R/Q (I8E4, Abcam), anti-CycT1 (10750, Santa Cruz Biotechnology) and anti-V5 (Invitrogen) antibodies have been purchased. A rabbit antibody against HEXIM1 was provided by Dr O Bensaude (Ecole Normale Supérieure, Paris, France). To detect 7SK, U2, MRP and U6 RNAs in Northern blotting, terminally labelled oligonucleotides complementary to the human 7SK (from U92 to G111, or from G272 to C291), U2 (from A28 to C50), MRP (from C185 to A206) or U6 (from U32 to G49) snRNAs were used.

In vitro reconstitution of 7SK snRNP and UV crosslinking

DNA templates for *in vitro* synthesis of internally labelled or cold 7SK, 7SKd1 to 7SKd7 and 7SKF1 to 7SKF4 RNAs were generated by PCR amplification by using the p7SK and p7SKd1 to p7SKd7 plasmids as templates and appropriate oligonucleotide primers incorporating the T7 promoter. *In vitro* RNA synthesis was performed with T7 RNA polymerase (Promega), in the presence or absence of [α-^{32}P]UTP (specific activity 30–40 Ci/mmol). The synthesized RNAs were purified on a 6% sequencing gel. About 10 fmol of labelled RNA was incubated with 2.5 µl of HeLa nuclear Dignam extract (corresponding to about 20 µg protein) (CilBiotech) in 25 µl of reconstitution buffer (100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF, 20 mM HEPES, pH 7.6) for 10 min at 30°C. After addition of 5 µg of *Escherichia coli* tRNA, the mixture was incubated for 5 min at 30°C and irradiated by 254 nm UV light for 5 min from an 8 cm distance, by using BIO-LINK® photocross-linker apparatus (Fisher Bioblock). RNAs were degraded by treatment with 2.5 µg of RNase A at 37°C for 30 min. Following SDS–PAGE and electrotransfer onto a nitrocellulose membrane (Hybond-C Extra, Amersham), the labelled proteins were detected by autoradiography.

Purification of 7SK RNP

Affinity selection of *in vitro* reconstituted 7SK RNPs was performed as described earlier (Wassarman and Steitz, 1991). Briefly, 15 µg of *in vitro* synthesized 7SK RNA was incubated with 750 pmol of biotinylated 2′-OMe RNA oligonucleotide (ABBBGACAGAUGUCG CAGCCA; B, denotes biotinylated 2′-deoxycytidine residues) for 1 h at 37°C in buffer A (100 mM NaCl, 5 mM $MgCl_2$ and 0.05% Nonidet P-40, 50 mM Tris–Cl, pH 8.0). About 50 µl of streptavidin agarose beads (Sigma) in buffer A were added and the mixture was rotated for 1 h at 4°C. The beads were washed three times with buffer A and incubated with 25 µl of HeLa nuclear extract (pretreated with 50 U of micrococcal nuclease for 1 h at 30°C) in 200 µl of reconstitution buffer (100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF, 20 mM HEPES, pH 7.6) supplemented with 40 U of RNasin (Promega) and protease inhibitor cocktail (complete EDTA-free, Roche). The beads were washed three times with buffer A and associated proteins were eluted by treatment with 1 µg RNase A for 1 h at 30°C. The eluted proteins were separated on a 8% SDS-polyacrylamide gel and stained with colloidal blue (Invitrogen).

For affinity selection of transiently expressed MS2-7SK RNP, about 1.6×10^8 HeLa cells transfected with the pMS2-7SK expression vector were suspended in 1 ml of NET2 buffer (200 mM NaCl, 0.05% Nonidet P-40, 20 mM Tris–HCl, pH 7.4) supplemented with 40 U of RNasin and protease inhibitor cocktail and sonicated three times for 30 s each with 1 min intervals using a Branson B15 sonifier at setting 3. The sonicate was cleared by centrifugation for 10 min at 15 000 *g*. Purified recombinant MS2-MBP protein (75 µg) was bound to 40 µl of amylose resin (New England BioLabs) in NET2 buffer, as described (Zhou *et al*, 2002). The beads were washed three times with 1 ml of NET2, before the sonic extract was added, and incubated at 4°C for 1 h. The beads were washed twice with NET2 and twice with low-salt buffer (10 mM NaCl, 0.5 mM EDTA, 2.0 mM DTT, 20 mM Tris–HCl, pH 7.4) and the associated MS2-7SK snRNP was eluted with 50 µl of low-salt buffer supplemented with 20 mM maltose.

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